PATENT

This application is a divisional of, and claims the benefit of priority from, U.S. Patent Application Serial No. 09/327,045, filed June 7, 1999, abandoned, the full disclosure of which is incorporated herein by reference in its entirety.

Please replace the paragraph beginning at page 27, line 2, with the following rewritten paragraph:

Preferred targeting molecules of the invention comprise an amino acid sequence selected from the group comprising GGGVFWQ, HGRVRPH, VVLVTSS, CLHRGNSC, and CRSWNKADNRSC (SEQ ID NO:1-5, respectively) using the *in vivo* panning procedure described above and referenced below. The GGGVFWQ , HGRVRPH, VVLVTSS, and CLHRGNSC (SEQ ID NO:1-4, respectively) peptides selectively bind to normal cardiac endothelium. More specifically, the GGGVFWQ (SEQ ID NO:1) peptide showed a 5-fold enrichment to normal cardiac vasculature, while the HGRVRPH, VVLVTSS, CLHRGNSC (SEQ ID NO:2-4, respectively) peptides showed a 2-fold enrichment to normal cardiac vasculature. The CRSWNKADNRSC (SEQ ID NO:5) peptide showed 5-fold enrichment to ischemic myocardium. Details of how these peptides were identified and their properties are described in U.S.S.N. 09/326,718 [Campbell & Flores LLP Attorney Docket # P-LJ 3512] filed on even date herewith which is specifically incorporated herein by reference.

Please replace the paragraph beginning at page 48, line 25, with the following rewritten paragraph:

The plasmid pVEGF-Bwt167 is constructed by insertion of a 580bp PCR product derived from phage Lambda gt11-VEGF-Bwt167 into the expression plasmid pSI (Promega, Inc.). This phage is obtainable by screening a human fibrosarcoma cDNA library in lambda g11 (obtainable from Clontech, Inc.). The PCR reaction is performed employing the Advantage KlenTaq Polymerase Mix system (Clontech. Inc.) in a final volume of 100 microliter containing lng of the plasmid template, 0.5μM of primers P-wt167(l) 5'-GATCGCTAGC GGCAGCATGA GCCCTCTGCT CCGCCGCCTG-3' (SEQ ID NO:6) and P-wt167(2) 5'-TGACGCGGCC GCTCACCTTC GCAGCTTCCG GCACCTGCAG-3' (SEQ ID NO:7) as well as 0.2mM dNTPs, using the conditions 93°C 30 sec, 55°C 30 sec, 72°C 30

sec for 30 cycles followed by a 72°C 10 min extension in a Pharmacia LKB Gene ATAQ Controller PCR. system. The PCR product is gel-purified, digested with NheI and NotI and ligated into the NheI/NotI cleaved plasmid pSI. The resulting plasmid is designated pVEGF-Bwt167.

Please replace the paragraph beginning at page 49, line 8, with the following rewritten paragraph:

EXAMPLE 2

Coupling of Peptide GGGVFWQ (SEQ ID NO:1) to VEGF-B_{167}

Principle

The N-terminally blocked peptide is activated at the C-terminus by the water soluble carbodiimide EDC (N-Ethyl-N'(3-dimethylaminopropyl) carbodiimide in the presence of N-hydroxysuccinimide (NHS). The activated peptide then reacts with the primary amino groups of the VEGF molecule. By adjusting the pH carefully it is possible to direct this reaction towards the N-terminus of the VEGF-B_{167} molecule (*see, e.g.*, Staros, J. *et al.*, *Anal. Biochem.* (1986) 156: 220-222 and Wong, S.S., "Application of Chemical Crosslinking to Soluble Proteins" in: CHEMISTRY OF PROTEIN CONJUGATION AND CROSSLINKING, (CRC Press Inc. 1993), pp. 221-229; these references and the references cited therein are incorporated herein by reference).

Please replace the paragraph beginning at page 50, line 5, with the following rewritten paragraph:

EXAMPLE 3

Coupling of a C-terminal elongated peptide GGGVFWQ (SEQ ID NO:1) to VEGF-B_{167}

Principle

To avoid sterical hindrance during the binding of VEGF-B chimeric molecule to the VEGF receptor resp. to the targeting peptide receptor the peptide can be elongated by several additional amino acids on the C-terminal end. The C-terminal spacer

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should allow maximal flexibility while not interfering in the binding mechanism of VEGF and/or peptide to their specific receptors. Usually poly-Gly or poly-Ala sequences fulfill these requirements.

Please replace the paragraph beginning at page 50, line 17, with the following rewritten paragraph:

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EXAMPLE 4

Coupling of peptide GGGVFWQ (SEQ ID NO:1) to VEGF-B_{167} by using a heterobifunctional reagent with a spacer domain

Principle

The coupling of the peptide can also be performed by reacting the N-terminus of the peptide with the amine-reactive part of a heterobifunctional crosslinker (for example SMBP), whereupon the activated peptide then reacts with an accessible sulfhydril group of VEGF-B_{167} to form a thioether linkage (*see, e.g.*, Staros, J. *et al.*, *Methods Enzymol.* (1989) 172, 609 and Wong, S.S., "Application of Chemical Crosslinking to Soluble Proteins" in: CHEMISTRY OF PROTEIN CONJUGATION AND CROSSLINKING, (CRC Press Inc. 1993), pp. 221-229). In the case of using SMBP the length of the spacer is in the order of 1.5nm. It has to be kept in mind that the sulfhydril group involved in the coupling reaction is not essential for the binding to the receptor protein.

Please replace the paragraph beginning at page 51, line 9, with the following rewritten paragraph:

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EXAMPLE 5

Non-covalent coupling of peptide GGGVFWQ (SEQ ID NO:1) to VEGF-B_{167}

Principle

Ionic interaction is one of the dominant forces in forming protein structures. By introducing regions of opposite charge into macromolecules it is possible to form tight complexes between two reaction partners which are also stable under physiological

conditions. The introduction of these charged amino acids has to be compatible with the function of both molecules.

Please replace the paragraph beginning at page 51, line 17, with the following rewritten paragraph:

Method

The peptide GGGVFWQ (SEQ ID NO:1) has to be modified at the N- or C-terminus by a stretch of 4-6 charged amino acids (Lysine, Arginine for the introduction of positive charges, Glutamic or Aspartic acid for the introduction of negative charges). Also the VEGF-B_{167} has to be extended preferably at the N-terminus with a sequence of 4-6 charged amino acids. Once the reaction partners are synthesized and purified to the appropriate degree of quality, the complexes can be formed easily just by mixing the equivalent amounts of the opposite charged reaction partners. Separation of unreacted molecules from conjugates can be performed using Ion Exchange Chromatography.

Please replace the paragraph beginning at page 52, line 1, with the following rewritten paragraph:

EXAMPLE 6

Conjugation of VEGF-B_{167} to a His-tagged peptide GGGVFWQ (SEQ ID NO:1)

Principle

In the case a complete separation of the VEGF chimeric molecule from free VEGF-B_{167} is necessary, the peptide can be elongated on the N- or C-terminal end with a stretch of 4-6 Histidine molecules. The coupling reaction is then performed according to example 2 or 5. For the capture of VEGF-B chimeric molecules, the approach of metal affinity chromatography can be used (Porath, J. *et al.*, *Nature* (1975) 258: 598-599).

Please replace the paragraph beginning at page 52, line 16, with the following rewritten paragraph:

EXAMPLE 7

Coupling of Peptide CRSWNKADNRSC (SEQ ID NO:5) to VEGF-B_{167}

In addition to the amino and carboxyl group of the N- and C-terminus, this peptide has two functional sulfhydril groups and one -amino group of Lysine that can be used for the coupling to VEGF-B_{167}. If it is necessary to use the peptide in a cyclic structure, only the amino- and carboxyl groups are available. Because there are more reactive groups on the peptide, the amount of theoretical byproducts can increase.

Please replace the paragraph beginning at page 53, line 6, with the following rewritten paragraph:

EXAMPLE 8

Carboxy-terminal (Ct) fusion of the targeting peptides GGGVFWQ (SEQ ID NO:1) and CRSWNKADNRSC (SEQ ID NO:5) to VEGF-B_{167}

Construction of plasmids pVEGF(B)-$(G_4S)_3$,-GGGVFWQ and pVEGF(B)-$(G_4S)_3$-CRSWNKADNRSC and expression of the chimeric molecules in CHO cells

The plasmids pVEGF(B)-$(G_4S)_3$-GGGVFWQ and pVEGF(B)-$(G_4S)_3$-CRSWNKADNRSC contain the DNA sequences coding for the targeting peptides NH_2-GGGVFWQ-COOH (SEQ ID NO:1) and NH_2-CRSWNKADNRSC-COOH (SEQ ID NO:5), respectively, fused to the C-terminus of the VEGF-B_{167} molecule via a NH_2-$(G_4S)_3$ –COOH (SEQ ID NO:8) hinge region. This type of linker is usually used to flexibly connect heavy and light chains in a single chain antibodies; alternatively, other connecting peptides, such as the natural hinge region present, in human immunoglobulin genes or oligo-proline or oligo-glycine linkers can be used. The linker peptide can, in addition, contain a protease cleavage site located between C-terminus of VEGF-B_{167} and the linker (*e.g.*, a plasmin cleavage site) allowing, after high affinity targeting to normal or ischemic heart, the release of a native VEGF-B_{167} molecule. Due to the flexibility of the linker, the C-terminal fusion peptide does not interfere with receptor binding.

Please replace the paragraph beginning at page 53, line 23, with the following rewritten paragraph:

A series of modular plasmids are constructed to finally obtain plasmids pVEGF(B)-$(G_4S)_3$-GGGVFWQ and pVEGF(B)-$(G_4S)_3$-CRSWNKADNRSC (*see* Materials and Methods, below). The intermediate plasmid pvegf-ss(l) provides the VEGF-B signal sequence followed by a HincII restriction site allowing for the convenient insertion of either the wild-type VEGF-B_{167} sequence or any other desired N-terminal fusion peptide (*see* Example 'N-Terminal fusions'). The final constructs, the plasmids pVEGF(B)-(G_4S)3-GGGVFWQ and pVEGF(B)-$(G_4S)_3$-CRSWNKADNRSC, are transfected into CHO cells. Cotransfection with a selectable marker, selection of CHO cell clones and production of the proteins can be carried out using standard cell culture and biotechnology procedures (*see, e.g.*, Example 1). The purification of the chimeric proteins is performed according to standard protein chemistry procedures (chromatography using anion and/or cation exchange resins, gel filtration or affinity chromatography).

Please replace the paragraph beginning at page 54, line 5, with the following rewritten paragraph:

Construction of plasmids

pSI-vegf-MCS(1)

In a first step the commercially available vector pSI (Promega) is cut with BglII treated with Klenow Polymerase using standard conditions and religated. The resulting intermediate plasmid is designated pSI-B. Subsequently, pSI-B is digested with NheI and NotI and ligated with annealed oligonucleotides P-vegfMCS(l) 5'- CTAGTACGTA TCTAGAGTCG ACACTAGTAG ATCTGATATC GCTAGCCTCG AGGCGGCGC CACGTGTACG TAGGCC-3' (SEQ ID NO:9), and P-vegfMCS(2) 5'- GGCCTACGTA CACGTGGCGG CCGCCTCGAG GCTAGCGATA TCAGATCTAC TAGTGTCGAC TCTAGATACG TA-3' (SEQ ID NO:10). The resulting plasmid is sequenced employing the primer P-4371 (5'-AATACGACTCACTATAG-3' (SEQ ID NO:11)) and designated pS1-vegf-MCS(l). pvegf-ss(1). Insertion of a DNA stretch encoding the VEGF-B_{167} signal sequence

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Met^{1}-Ala^{21} including amino acid codons Pro^{22}, Val^{23} and Asp^{27} is done by ligating the XbaI/SalI cut vector pSI-vegf-MCS(1) with the annealed oligonucleotides P-ss(l) 5'-CTAG GCCACCATGAGCC CTCTGCTCCG CCGCCTGCTG CTCGCCGCAC TCCTGCAGCT GGCCCCCGCC CAGGCCCCTG -3' (SEQ ID NO:12) and P-ss(2) 5'- TCGACAGGGG CCTGGGCGGG GGCCAGCTGC AGGAGTGCGG CGAGCAGCAG GCGGCGGAGC AGAGGGCTCA TGGTGGC-3' (SEQ ID NO:13). The inserted region is sequenced (primer P-4371) and the resulting plasmid 15 named pvegf-ss(1). Amino acid codons Val^{23} and Asp^{27} form a HincII restriction site. This allows for the convenient insertion of either the wildtype $VEGF\text{-}B_{167}$ sequence (codons Ser^{24} Gln^{25} and Pro^{26}) or for any desired N-terminal fusion peptide.

Please replace the paragraph beginning at page 54, line 26, with the following rewritten paragraph:

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pvegf-d24/26

In order to construct the vector pvegf-d24/26, $VEGF\text{-}B_{167}$ coding sequences corresponding to amino acid residues Asp^{27} to Arg^{188} are amplified as a 500bp PCR product in a standard PCR reaction employing primers $2\text{-}27/_{167}(1)$ 5'- GATCGTCGAC GCCCCTGGCC ACCAGAGGAA AGTGG -3' (SEQ ID NO:14) and $P\text{-}27/_{167}(2)$ 5'- GATCAGATCT TCGCAGCTTC CGGCACCTGC AGGTG -3' (SEQ ID NO:15). The PCR product is digested with SalI/Bg1ll and the resulting 486bp fragment is cloned into SalI/BglII cut plasmid pvegf-ss(1).

Please replace the paragraph beginning at page 55, line 14, with the following rewritten paragraph:

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pVEGF(B)-$(G_4S)_3$

To complete the construction of the vector pVEGF(B)-$(G_4S)_3$, annealed oligonucleotides P-Li(1) 5'- GATCTGGCGG CGGCGGCAGC GGCGGCGGCG GCAGCGGCGG CGGCGGCTCT G-3' (SEQ ID NO:16), and P-Li(2) 5' CTAGCAGAGC CGCCGCCGCC GCTGCCGCCG CCGCCGCTGC CGCCGCCGCC A-3' (SEQ ID NO:17)

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encoding the $(G_4S)_3$ (SEQ ID NO:8) linker sequence, are inserted into Bg1II/NheI cut vector pVEGF(B)-F.

Please replace the paragraph beginning at page 55, line 21, with the following rewritten paragraph:

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pVEGF(B)$(G_4S)_3$-GGGVFWQ

Construction of pVEGF(B)-$(G_4S)_3$-GGGVFWQ is done by ligation of NheI/NotI cut vector pVEGF(B)-$(G_4S)_3$, with annealed oligonucleotides P-D(1) 5'- CTAGC GGC GGG GGC GTG TTC TGG CAG TAAGC-3' (SEQ ID NO:18), and P-D(2) 5'- GGCCGCTT ACTGCCAGAA CACGCCCCCG CCG-3' (SEQ ID NO:19). The plasmid pVEGF(B)-$(G_4S)_3$-GGGVFWQ contains the DNA sequences coding for the targeting peptide NH_2-GGGVFWQ-COOH (SEQ ID NO:1) fused to the C-terminus of the VEGF-B_{167} cDNA via a NH_2-$(G_4S)_3$-COOH (SEQ ID NO:8) hinge region.

Please replace the paragraph beginning at page 55, line 29, with the following rewritten paragraph:

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pVEGF(B)-$(G_4S)_3$-CRSWNKADNRSC

Construction of pVEGF(B)-$(G_4S)_3$-CRSWNKADNRSC was done by ligation of NheI/NotI cut vector pVEGF(B)-$(G_4S)_3$, with annealed oligonucleotides P-CRSWNKADNRSC(1) 5'-CTAGCTGCC GCAGCTGGAA CAAAGCCGAC AACCGCAGCT GCTAAGC-3' (SEQ ID NO:20) and P-CRSWNKADNRSC(2) 5'-GGCCGCTT AGCAGCTGCG GTTGTCGGCT-3' (SEQ ID NO:21).

Please replace the paragraph beginning at page 56, line 4, with the following rewritten paragraph:

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EXAMPLE 9

Amino-terminal (Nt) fusion of the targeting peptide CRSWNKADNRSC (SEQ ID NO:5) to VEGF-B_{186}

Construction of the plasmid pVEGF(B)-Nt-CRSWNKADNRSC and expression of the chimeric molecules in CHO cells

The plasmid pVEGF(B)-Nt-CRSWNKADNRSC contains the DNA sequences coding for the heart tissue target peptide NH_2-CRSWNKADNRSC-COOH (SEQ ID NO:5) inserted between the signal peptide and the N-terminus of the VEGF-B_{186} molecule via a NH_2-$(G_4S)_3$-COOH (SEQ ID NO:8) hinge region. Other linker peptides containing functional elements may be used (*see* Example 8 above). The N-terminal fusion allows the natural proteolytic processing occurring with the VEGF-B_{186} molecule without loss of the targeting molecule. Since the N-terminus appears to be located distal to the membrane binding face of the dimeric VEGF molecule, the fused targeting peptide can interact without steric hindrance with its receptor. Part of the series of modular plasmids described in example 8 is used to further construct the plasmid pVEGF(B)-Nt- CRSWNKADNRSC (*see* Materials and Methods). The final construct is transfected into CHO cells. Cotransfection with a selection marker, selection of CHO cell clones and production of protein is carried out using standard cell culture and biotechnology procedures (*see* Example 1). The purification of the chimeric proteins is done according to standard protein chemistry procedures.

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Please replace the paragraph beginning at page 56, line 24, with the following rewritten paragraph:

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Materials and Methods

Construction of plasmids

pVEGF(B)186-d24/26

Construction of pVEGF(B)186-d24/26 is done by digestion of pvegf-d24/26-dH (*see* Example 8) with SalI and BglII. A 492bp fragment is removed by gel purification. This step deletes DNA sequences coding for amino acids Asp27 to Arg188 of VEGF(B)167 from plasmid pvegf-d24/26-dH (*see* Example 8). Subsequently a 553 bp SalI/BglII cut PCR product coding for amino acid Asp^{27}-Ala^{207} of VEGF(B)186 is inserted. PCR is done as a standard PCR reaction employing primers P-27/167(1) and P-27/186(1) (5'-

TGACAGATCT CTAAGCCCCG CCCTTGGCAA CGGAGG-3') (SEQ ID NO:22) and VEGF(B)186 cDNA as a template. In the final plasmid pVEGF(B)186-d24/26 amino acids Asp^{27} to Arg^{188} of VEGF(B)167 are replaced by amino acids Asp^{27} to Ala^{207} of VEGF(B)186, amino acids Met^{1} to Val^{23} are common to both VEGF(B) forms whereas amino acids Ser^{24}, Gln^{25} and Pro^{26} are still missing.

Please replace the paragraph beginning at page 57, line 7, with the following rewritten paragraph:

pVEGF(B)186-Nt-R13

Construction of pVEGF(B)186-Nt-CRSWNKADNRSC is done by ligating HindII cleaved vector pVEGF(B)186-d24/26 with annealed oligonucleotides P-Nt-CRSWNKADNRSC(1) 5'- TGCCGCAGCT GGAACAAAGC CGACAACCGC AGCTGCTCCC AGCCT-3' (SEQ ID NO:23) and P-Nt-CRSWNKADNRSC(2) 5'-AGGCTGGGAG CAGCTGCGGT TGTCGGCTTT GTTCCAGCTG CGGCA-3' (SEQ ID NO:24). The plasmid containing the oligonucleotides inserted into the opposite direction is also isolated and designated pVEGF(B)186-Nt-antisense.

Please insert the accompanying paper copy of the Sequence Listing, page numbers 1 to 7, at the end of the application.

REMARKS

Applicants request entry of this amendment in adherence with 37 C.F.R. §§1.821 to §1.825. In accordance with 37 CFR §1.821 (e)This amendment is accompanied by a floppy disk containing the above named sequences, SEQ ID NOS:1-24, in computer readable form, and a paper copy of the sequence information which has been printed from the floppy disk.

The specification has been also amended to insert a reference to the priority application.